



TITLE:

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**Absence of a gene encoding cytosine deaminase in the genome of the agaricomycete  
*Coprinopsis cinerea* enables simple marker recycling through 5-fluorocytosine  
counter-selection**

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## Abstract

*Coprinopsis cinerea* is a model species for molecular genetics studies of sexual development in agaricomycetes or homobasidiomycetes. Recently, efficient gene targeting was established in this fungus by generating *Cc.ku70* or *Cc.lig4* disruptants. To determine the molecular mechanisms underlying sexual development, which involves many genes, generating multiple gene disruptants is required. However, the number of transformation markers available for *C. cinerea* is limited. This problem would be solved by establishing marker recycling. In this study, we found that *C. cinerea* lacks a gene encoding a homolog of *Saccharomyces cerevisiae* cytosine deaminase (Fcy1p) in its genome, which is present in many other fungi. We also observed that *C. cinerea* is resistant to 5-fluorocytosine. Based on these findings, we established a simple marker recycling method in this fungus using 5-fluorocytosine counter-selection after heterologous expression of FCY1 derived from *Pleurotus ostreatus*, together with the hygromycin resistance gene. This study proposes a simple genetic manipulation system that can be performed using wild-type strains of several fungi that lack a gene homologous to *S. cerevisiae* FCY1 in their genomes.

67     **Introduction**

68                    The inky cap mushroom *Coprinopsis cinerea* has been used for classical and  
69     molecular genetics studies of fungal multicellular development, fruiting-body or mushroom  
70     formation, for many years because it completes its life cycle in 2 weeks and produces abundant  
71     oidia, which are uninucleate asexual spores (for reviews, see Kües 2000; Kamada 2002). Its  
72     easy and reliable genetic transformation system (Granado *et al.* 1997; Cummings *et al.* 1999; Ito  
73     *et al.* 2004), useful tools for genetic analysis (Zolan *et al.* 1992; Muraguchi *et al.* 2003) and  
74     carefully assembled genomic sequence (Stajich *et al.* 2010) also make it very easy to conduct  
75     molecular genetic studies in *C. cinerea* through forward genetics. However, gene targeting  
76     through homologous recombination to disrupt or modify genes of interest is challenging in this  
77     fungus, because the frequency of homologous recombination is very low, almost 0%. This has  
78     been a serious bottleneck in *C. cinerea* studies. Recently, Nakazawa *et al.* (2011) established  
79     high-frequency gene targeting in *C. cinerea* by generating strains in which *Cc.ku70* or *Cc.lig4*  
80     are disrupted.

81                    Efficient gene targeting in other agaricomycetes, such as *Schizophyllum commune*  
82     and *Pleurotus ostreatus*, has also been established (de Jong *et al.* 2010; Salame *et al.* 2012).  
83     However, it remains difficult to generate strains in which multiple genes are modified or  
84     disrupted, because the number of transformation markers available in these fungi is limited.  
85     Therefore, a marker recycling system should be developed in agaricomycetes. In various  
86     filamentous fungi, marker recycling has been established by 5-fluoroorotic acid (5-FOA)  
87     counter-selection, which is carried out after auxotrophic complementary transformation of *pyrG*  
88     mutants (Boeke *et al.* 1984; Yoon *et al.* 2011; Nakazawa *et al.* 2013). However, mutations in

*pyrG* generally cause uridine/uracil auxotrophy. Even when *pyrG* mutants are cultured on complete media, such as yeast and malt extract with glucose (YMG) medium, it is often necessary to supply a large amount of uridine for their hyphal growth, which might cause unexpected physiological and biochemical effect(s) in the fungus and make it difficult to examine the precise effects of gene disruption on the phenotype of interest. Therefore, it is desirable to establish a different marker recycling system.

The *FCYI* gene, which encodes cytosine deaminase, has been used frequently for 5-fluorocytosine (5-FC) counter-selection in yeasts (Jund *et al.* 1970; Erbs *et al.* 1997). As far as we know, mutations in *FCYI* have never been reported to cause auxotrophy in yeast. Furthermore, *in silico* analysis suggested that wild-type *C. cinerea* lacks a gene homologous to yeast *FCYI* in its genome, suggesting that generating an *fcyI* mutant sensitive to 5-FC, which is a prerequisite for 5-FC counter-selection, would not be required. Therefore, this counter-selection system would be more suitable in this fungus.

## Materials and Methods

*Strains, culture conditions and genetic techniques of C. cinerea and P. ostreatus*

The *C. cinerea* and *P. ostreatus* strains used in this study are listed in Table 1. YMG medium (Rao and Niederpruem 1969) solidified with 2% (w/v) agar in 4- or 9-cm Petri dishes was used for routine cultures. Cultures were maintained at 28°C under continuous darkness. For fruiting and oidia production, *C. cinerea* strains were cultured on YMG slants or YMG agar plates at 28°C under a 12-h light/12-h dark cycle. Crosses were performed as described by Inada *et al.* (2001). In this study, transformation was performed as described by Nakazawa *et al.*

(2010), using protoplasts prepared from mycelial cells.

### *Construction of plasmids to express PoFCY1 in C. cinerea*

To express *P. ostreatus* FCY1 (*PoFCY1*) under the control of the *C. cinerea*  $\beta 1$ -*tub* promoter, which has been the most frequently used promoter for heterologous expression of proteins in this fungus (Cummings *et al.* 1999; Nakazawa *et al.* 2009; Muraguchi *et al.* 2011), we performed inverse polymerase chain reaction (PCR) using pPHT1 (Cummings *et al.* 1999) as a template and the primer set TN86-TN88 to yield linear DNA in which *hph*, the gene encoding hygromycin phosphotransferase (conferring resistance to hygromycin B), was deleted from pPHT1. The cDNA fragment of *Pofcy1* (from the predicted translation start site to the stop site shown in the genome web site described in the Results and discussion) was also amplified by reverse-transcription (RT)-PCR. In this study, total RNAs from *P. ostreatus* PC9 were reverse-transcribed using SuperScript III Reverse Transcriptase (Life Technologies, CA, USA), followed by a conventional PCR reaction using the primer set TN84-TN85, to amplify the cDNA fragment of *Pofcy1*. The resulting two DNA fragments were fused using a Geneart Seamless Cloning and Assembly kit (Life Technologies, CA, USA). We designated the resulting plasmid pTN2000. To construct a plasmid to express HPH fused with *PoFCY1*, we performed inverse PCR using pPHT1 as a template and the primer set TN87-TN88. The amplified linear DNA was fused with the cDNA fragment of *Pofcy1* prepared above using the Geneart Seamless Cloning and Assembly kit: the resulting plasmid was designated pTN2001.

To amplify the genomic fragment containing a putative open reading frame (ORF) of *Pofcy1*, together with its 5'-upstream and 3'-downstream sequences, we performed PCR with

genomic DNAs from strain PC9 as a template and the primer set TN101-TN102. The amplified genomic fragment was cloned into pBluescript II KS+ digested with *EcoRV*. The resulting plasmid was digested with *HindIII* and *EcoRI*, followed by reaction with the Klenow Fragment (Takara Bio, Shiga, Japan). This DNA fragment, containing the *PofcyI* gene, was inserted into pPHT1 digested with *EcoRV* to yield plasmid pTN2002, which contained the expression cassette for HPH from plasmid pPHT1 and the *PofcyI* gene from strain PC9. Next, pTN2000 was digested with *EcoRI* to obtain the expression cassette for *PoFCY1* under the control of the *C. cinerea*  $\beta 1$ -*tub* promoter. The obtained DNA fragment was treated with the Klenow Fragment and inserted into pPHT1 digested with *EcoRV* to yield plasmid pTN2003, which contained expression cassettes for HPH and *PoFCY1* from pPHT1 and pTN2000, respectively. The *C. cinerea*  $\beta 1$ -*tub* promoter from plasmid pPHT1 and the genomic fragment containing the putative ORF of *PofcyI* and its 3'-downstream region from *P. ostreatus* strain PC9 were amplified by PCR using primer sets TN103-TN86 and TN84-TN102, respectively. They were then fused by overlap extension PCR. The resulting expression cassette for *PoFCY1* was inserted into pPHT1 digested with *EcoRV* after treatment with T4 Polynucleotide Kinase (Takara Bio, Shiga, Japan) to yield plasmid pTN2004.

A DNA fragment containing the 3'-downstream sequence of *PofcyI* was amplified by PCR using the primer set TN114-TN115 (the template was pTN2004). The resulting DNA fragment (351 bp) and plasmid pTN2004 were both digested with *BamHI* and *HindIII*, and then fused to yield plasmid pTN2005, which contained two direct repeat sequences (the 3'-downstream sequence of *PofcyI*) that were there to mediate intramolecular homologous recombination to remove the expression cassettes for HPH and *PoFCY1* contained in plasmid

pTN2005 (Fig. 4A).

### *Construction of the Cc.mpr1-disrupting plasmid*

A genomic fragment (Scaffold 4: 373685–377348), amplified using the genomic DNA from ku3-24 as a template and primer set TN120-TN123, was cloned into pBluescript II KS+ digested with *EcoRV*. Inverse PCR was performed with the resulting plasmid as a template and the primer set TN121-TN122. A DNA fragment containing the expression cassettes for HPH and *PoFCY1* was also amplified by PCR using pTN2005 as the template and the primer set M13F-M13R. The resulting two DNA fragments were fused using the Geneart Seamless Cloning and Assembly kit to yield a plasmid containing the *Cc.mpr1*-disruption cassette. The protein ID used to search and obtain sequence data for *Cc.mpr1* (and the protein encoded by it) from the genome database described in the Results and discussion was 469410.

### *Colony PCR and genomic PCR*

We carried out colony PCR as described by Nakazawa *et al.* (2011) for rapid screening for gene disruptants and strains from which markers *hph* and *Pofcy1* were removed, except that the EmeraldAmp MAX PCR Master Mix (Takara Bio, Shiga, Japan) was used in this study. After colony PCR, we then performed genomic PCR. Genomic DNAs were extracted as described by Zolan and Pukkila (1986) and Muraguchi *et al.* (2003).

### *5-FC counter-selection*



Strain mpr-3 was cultured on a YMG agar plate (9-cm Petri dishes) under a 12-h light/12-h dark cycle for 9 days at 28°C. Oidia ( $10^7$ ) harvested from mpr-3 were spread onto a YMG agar plate containing 0.1% (w/v) 5-FC. After 4–6 days, 5-FC resistant colonies appeared on the agar medium. They were transferred onto a YMG agar plate containing 0.1% (w/v) 5-FC only once for nuclear purification. After this passage, each of the obtained clones, rev#2–7, was inoculated onto YMG agar medium containing 100  $\mu$ g/ml (w/v) hygromycin-B to examine whether they were sensitive or resistant to hygromycin-B. DNA analyses by colony PCR and genomic PCR was then carried out as above.

## Results and Discussion

### *C. cinerea* wild-type strains exhibited resistance to 5-FC

We found that *C. cinerea* does not possess a gene encoding a homolog of cytosine deaminase in its genome ([http://genome.jgi.doe.gov/Copci\\_AmutBmut1/Copci\\_AmutBmut1.home.html](http://genome.jgi.doe.gov/Copci_AmutBmut1/Copci_AmutBmut1.home.html)), whereas *P. ostreatus* does (protein ID 89004 In: JGI [http://genome.jgi-psf.org/PleosPC9\\_1/PleosPC9\\_1.home.html](http://genome.jgi-psf.org/PleosPC9_1/PleosPC9_1.home.html), identity = 60% (90/150), *E* value =  $9e-61$ ). Two *C. cinerea* wild-type strains, 326 and KF<sub>3</sub>#2, and *P. ostreatus* strain PC9 were assayed for sensitivity or resistance to 5-FC. The two *C. cinerea* strains were resistant to 5-FC, whereas *P. ostreatus* strain PC9 was sensitive (Fig. 1), which was consistent with the presence or absence of a gene homologous to yeast *FCY1*. This finding prompted us to examine whether heterologous expression of *P. ostreatus* FCY1, *PoFCY1*, confers sensitivity to 5-FC on *C. cinerea*.

199

200 *Expressing PoFCY1 in C. cinerea strain 326 conferred sensitivity to 5-FC*

201 Plasmid pTN2000, carrying the expression cassette for *PoFCY1*, was introduced into  
202 *C. cinerea* strain 326. pTN2000 was co-transformed with pPHT1 carrying the transformation  
203 marker *hph* (Cummings *et al.* 1999). Eight out of 44 strains obtained as hygromycin-B resistant  
204 transformants did not grow on YMG agar medium containing 0.1% (w/v) 5-FC, whereas they  
205 grew on YMG agar medium without it. When strain 326 was transformed with plasmid pPHT1  
206 only, as a control, all of the obtained hygromycin-B resistant transformants (37 strains) were  
207 resistant to 5-FC (Fig. 2A), supporting the hypothesis that heterologous expression of cytosine  
208 deaminase derived from *P. ostreatus* in *C. cinerea* conferred sensitivity to 5-FC.

209

210 *Examination of constructs conferring sensitivity to 5-FC on C. cinerea*

211 Plasmid pTN2001 (Fig. 2A) was introduced into strain 326 to express HPH fused  
212 with *PoFCY1*. However, the number of transformants obtained by introducing pTN2001 was  
213 lower than that obtained by introducing plasmid pPHT1, and all 18 of the obtained  
214 transformants, which were isolated as hygromycin-B resistant strains, were resistant to 5-FC  
215 (Fig. 2A). This suggested that the fusion protein lost its activity and did not function as expected.  
216 We then transformed strain 326 with plasmid pTN2002 containing the *Pofcy1* gene from *P.*  
217 *ostreatus* strain PC9 (Fig. 2A and B), which resulted in only two out of the 67 hygromycin-B  
218 resistant transformants exhibiting sensitivity to 5-FC. Next, we examined plasmid pTN2003.  
219 This plasmid contains expression cassettes for HPH and *PoFCY1* from pPHT1 and pTN2000,  
220 respectively, both of which are expressed under the control of the *C. cinerea*  $\beta 1$ -*tub* promoter

(Fig. 2A). Among 21 hygromycin-B resistant transformants obtained after introducing plasmid pTN2003, 14 strains were sensitive to 5-FC (Fig. 2A and B). Furthermore, plasmid pTN2004 (Fig. 2A) was constructed as described in Materials and Methods, and was introduced into strain 326, resulting in 20 out of 33 hygromycin-B resistant transformants being sensitive to 5-FC (Fig. 2A). The fact that plasmid pTN2002 did not confer sensitivity to 5FC on strain 326 efficiently, whereas plasmids pTN2003 and pTN2004 did, suggested that the *Pofcy1* promoter derived from *P. ostreatus* strain PC9 did not function in *C. cinerea* or could not express sufficient mRNA to confer sensitivity to 5-FC on strain 326.

Based on these results, we created plasmid pTN2005 so that marker recycling through 5-FC counter-selection would be demonstrated after gene targeting experiments (see Materials and Methods and Fig. 4A).

### *Targeted disruption of Cc.mpr1 encoding a putative acetyltransferase similar to Saccharomyces cerevisiae Mpr1*

We used strain ku3-24, which was newly generated in this study (Table 1), instead of 326 for efficient gene targeting experiment (Nakazawa *et al.* 2011). We also confirmed that strain ku3-24 was resistant to 5-FC like strains 326 and KF<sub>3</sub>#2 (data not shown).

We transformed *C. cinerea* strain ku3-24 with the *Cc.mpr1*-disrupting construct created as described in Materials and Methods to replace the entire ORF of *Cc.mpr1* encoding a putative acetyltransferase homologous *S. cerevisiae* Mpr1p (sigma1278b gene for L-proline-analogue resistance) (identity = 39% (73/183), *E* value = 3e-32) (Kimura *et al.* 2002) with the construct contained in plasmid pTN2005 (Figs. 3A and 4A). Two *Cc.mpr1* disruptants,

mpr-1 and mpr-3, both of which exhibited sensitivity to 5-FC, were obtained (Fig. 3B). mpr-2 was also shown to possess nuclei in which *Cc.mpr1* was disrupted (the left part of Fig. 3B). However, this strain exhibited slight resistance to 5-FC (data not shown), suggesting nuclear purification was insufficient. Genomic PCR using the primer set TN144-TN145 also suggested that mpr-2 possesses nuclei in which *Cc.mpr1* was not disrupted (white arrow in the right part of Fig. 3B). Therefore, we did not consider this transformant as a *Cc.mpr1* disruptant.

#### *5-FC counter-selection to isolate a nucleus in which marker excision occurred*

Oidia harvested from strain mpr-3 were subject to 5-FC counter-selection as described in Materials and Methods, resulting in the isolation of six 5-FC resistant strains, rev#2–7. Two of the six strains, rev#2 and rev#5, exhibited sensitivity to hygromycin B, suggesting that the construct used for *Cc.mpr1* disruption was removed through intramolecular homologous recombination mediated by the two direct repeat sequences (thick arrows shown in Fig. 4A) in these two strains. Colony PCR was performed to examine whether marker excision had occurred (Fig. 4B). A 5.4-kb fragment was expected to be amplified from the genome in which the entire ORF of *Cc.mpr1* was replaced with the construct (before marker excision/5-FC counter selection, as shown in Fig. 4A), whereas a 2.1-kb fragment was expected to be amplified from the genome in which the construct contained in pTN2005 used for *Cc.mpr1* disruption was removed (after marker excision/5-FC counter selection, as shown in Fig. 4A). The results shown in Fig. 4B suggested that the construct has been successfully removed in strains rev#2 and rev#5. Both DNA fragments were amplified from strain rev#7, which suggested that this strain possessed both types of nuclei: those in which the construct was

removed and those that still retained the construct. This might indicate that the single passage used for nuclear purification was insufficient for this strain.

We then performed genomic PCR for strains rev#2, rev#5, mpr-3 and ku3-24 as a control (Fig. 4C), demonstrating that the construct contained in pTN2005 had been successfully removed in strains rev#2 and rev#5. Small amounts of 2.0- and 2.1- kb fragments, indicated by white arrows in Fig. 4C, which were expected to be amplified from the genome of strains in which the construct contained in pTN2005 used for *Cc.mpr1* disruption was removed (after marker excision/5-FC counter selection as shown in Fig. 4A), were also amplified from genomic DNAs from strain mpr-3. This showed that mpr-3 contained a small number of nuclei in which intramolecular homologous recombination had occurred, and that these nuclei were purified through 5-FC counter-selection.

There are another direct repeat sequences (the  $\beta 1$ -*tub* promoter used for expressions of both HPH and *PoFCY1*) in plasmid pTN2005, and the construct used for *Cc.mpr1* disruption (Fig. 4A) Therefore, intramolecular homologous recombination mediated by these sequences might also occur. However, nuclei in which such intramolecular homologous recombination had occurred were not isolated through 5-FC counter selection in this study. This might be because the nuclei retained the expression cassette for *PoFCY1*, despite the occurrence of intramolecular homologous recombination, which continued to confer sensitivity to 5-FC on *C. cinerea* strains.

## Conclusions

In this study, we presented a simple and useful marker recycling system in *C. cinerea*. This new genetic manipulation system, which does not cause any observable auxotrophy, could

be used as a means to create multiple gene disruptants for detailed genetic studies in this fungus.

Genome database searching suggested that some fungi, such as *Agaricus bisporus*, *Magnaporthe oryzae* and *Chaetomium globosum* also lack a gene encoding an Fcy1p homolog in their genomes. Therefore, marker recycling based on *fcy1/5*-FC counter-selection could be performed in these fungi, as well as in *C. cinerea*. It would also be interesting to investigate why these fungi lack a gene encoding cytosine deaminase.

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## Figure legends

**Fig. 1.** *C. cinerea* strains exhibited resistance to 5-FC. *C. cinerea* strains 326, KF<sub>3</sub>#2 and *P. ostreatus* strain PC9 were cultured on yeast and malt extract with glucose (YMG) agar plates with and without 0.1% (w/v) 5-fluorocytosine (5-FC). Strains 326 and KF<sub>3</sub>#2 were cultured at 28°C for 4 days. Strain PC9 was cultured at 28°C for 6 days. The scale bars represent 2 cm.

**Fig. 2.** Heterologous expression of *PoFCY1* in *C. cinerea* strain 326 conferred sensitivity to 5-FC. (A) Plasmids pTN2000–pTN2004 containing different expression cassettes for *PoFCY1* were used to confer sensitivity to 5-fluorocytosine (5-FC) on *C. cinerea* strain 326, “5-FC<sup>S</sup> / total transformants” indicates the number of transformants exhibiting sensitivity to 5-FC among all of the hygromycin-B resistant transformants obtained after introducing each plasmid. Small white bars shown in plasmids pTN2002 and pTN2004 indicate predicted introns. (B) An example of conferring sensitivity to 5-FC by heterologous expression of *PoFCY1* in *C. cinerea* strain 326. White arrows indicate 5-FC-sensitive transformants obtained after introducing plasmid pTN2003.

**Fig. 3.** Targeted disruption of *Cc.mpr1*. (A) A diagram of the genomic locus of *Cc.mpr1*. Black arrows indicate the primers used for the PCR experiments in (B). (B) Genomic PCR experiments confirming *Cc.mpr1* disruption. Lane 1, the parental strain ku3-24 as a control; Lane 2, *mpr-1*; Lane 3, *mpr-2*; Lane 4, *mpr-3*. Lanes M on the left and right sides indicate size markers:  $\lambda$ HindIII/EcoRI markers on the left and a 100 bp ladder marker (100–1000 bp) on the right.

397

398 **Fig. 4.** Isolation of a nucleus in which marker excision occurred through 5-fluorocytosine  
399 (5-FC) counter-selection, and PCR experiments confirming that it had occurred. (A) Diagram of  
400 the genomic locus of the *Cc.mpr1* gene in strain ku3-24, *Cc.mpr1* disruptants from ku3-24  
401 (before marker excision) and strains in which the construct used for *Cc.mpr1* disruption was  
402 removed by intramolecular homologous recombination (after marker excision). Thin arrows  
403 indicate the primers used for PCR. Thick arrows indicate direct repeat sequences that mediate  
404 intramolecular homologous recombination to remove expression cassettes for HPH and  
405 *PoFCY1*. (B) Colony PCR screening for strains in which the construct used for *Cc.mpr1*  
406 disruption was removed. Lane M,  $\lambda$ HindIII/EcoRI markers; #2-#7 (described as rev#2-rev#7 in  
407 the main text), 5-FC-resistant strains isolated after 5-FC counter-selection against strain mpr-3.  
408 Hyg “S” indicates a hygromycin-B sensitive strain, whereas “R” indicates a resistant strain. (C)  
409 Genomic PCR experiments confirming marker excision had occurred. Lane M, size markers  
410 ( $\lambda$ HindIII for the left side, and a 100 bp ladder marker (100–1000 bp) for the right side); Lane 1,  
411 rev#2; Lane 2, rev#5; Lane 3, mpr-3; Lane 4, ku3-24.

412

## Wild-type *C. cinerea*



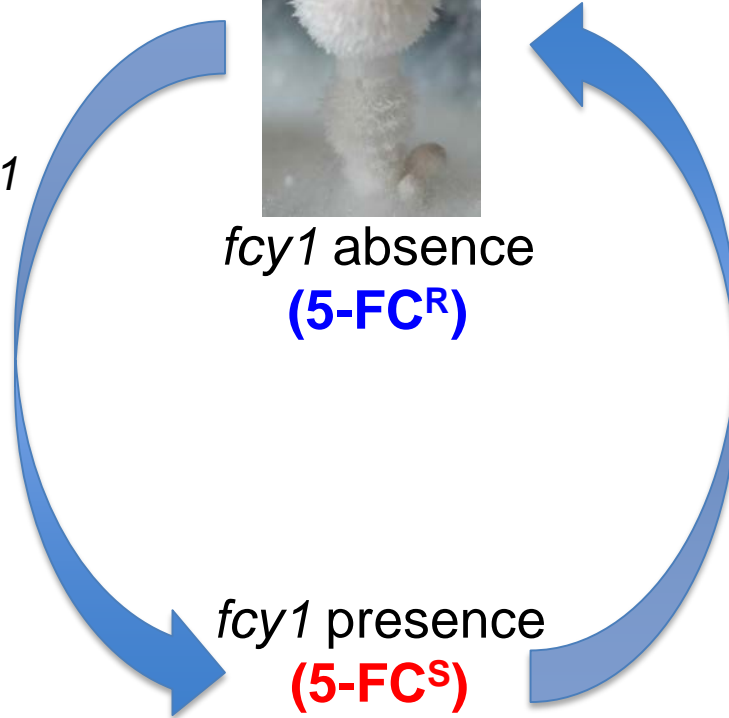
*P. ostreatus fcy1*



*fcy1* absence  
(5-FC<sup>R</sup>)

marker excision  
(5-FC countere-selection)

*fcy1* presence  
(5-FC<sup>S</sup>)



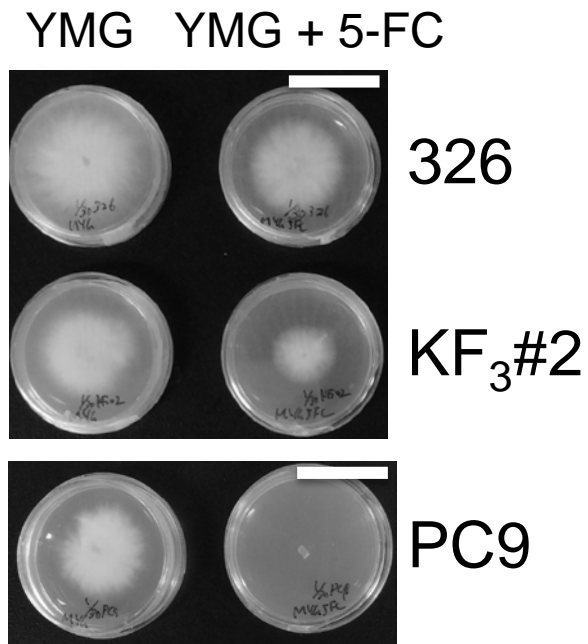
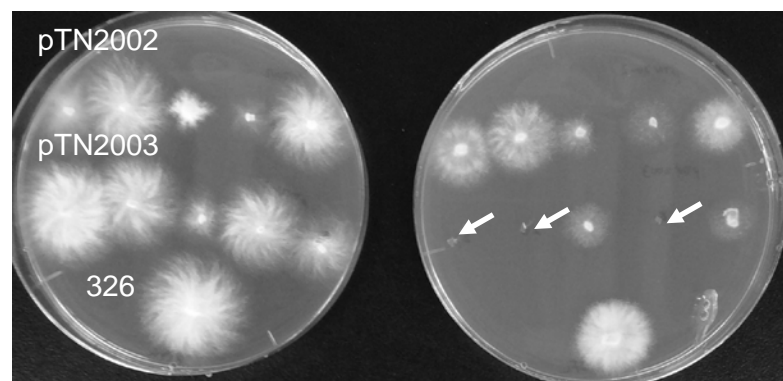
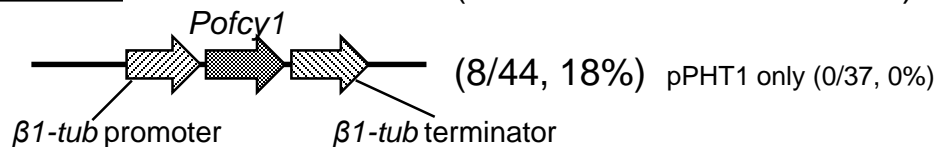


Fig. 1. Nakazawa et al.

B

pTN2000

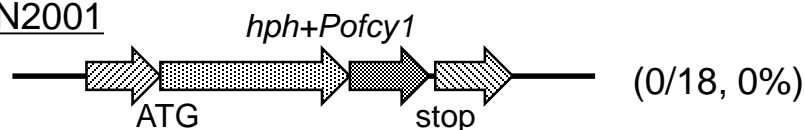
(5-FC<sup>S</sup> / total transformants)



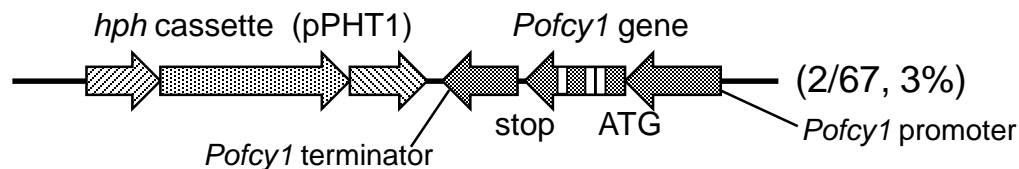
YMG

YMG + 5-FC

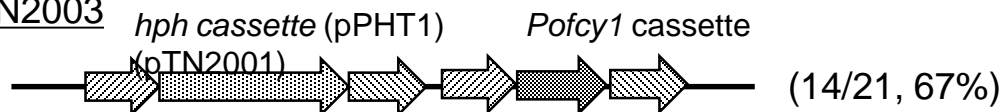
pTN2001



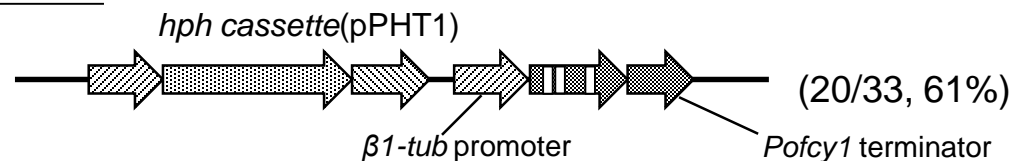
pTN2002



pTN2003



pTN2004



1 kb

Fig. 2. Nakazawa et al.

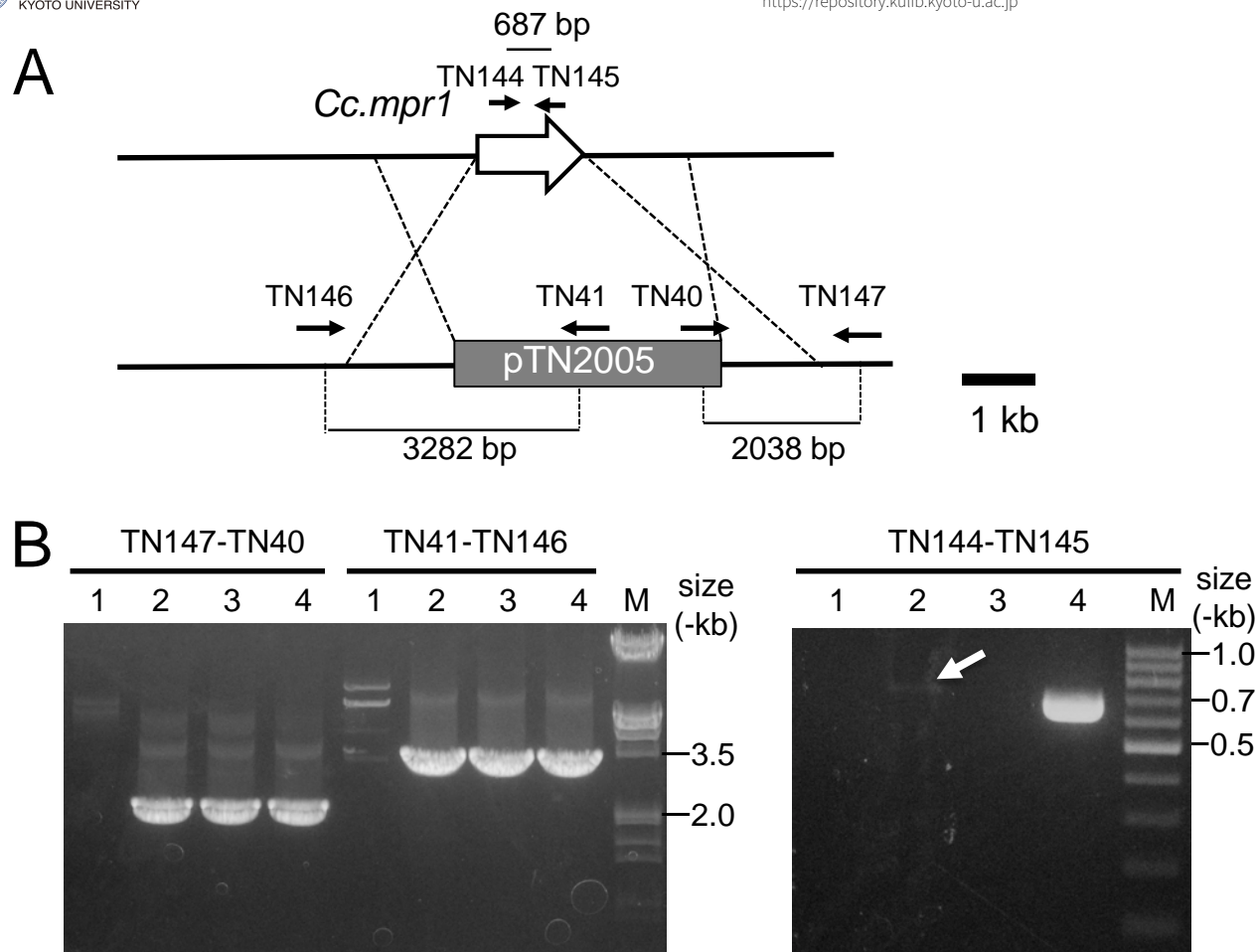


Fig. 3. Nakazawa et al.

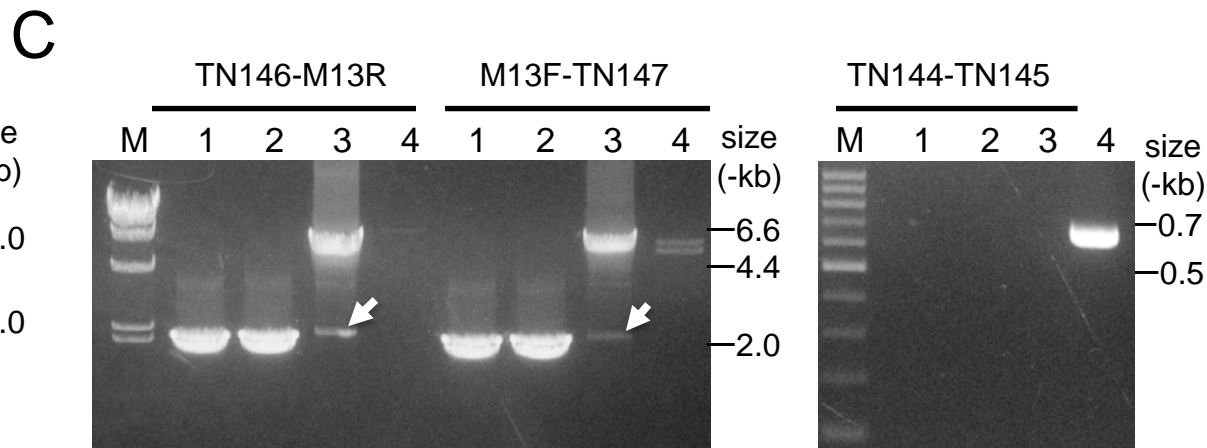
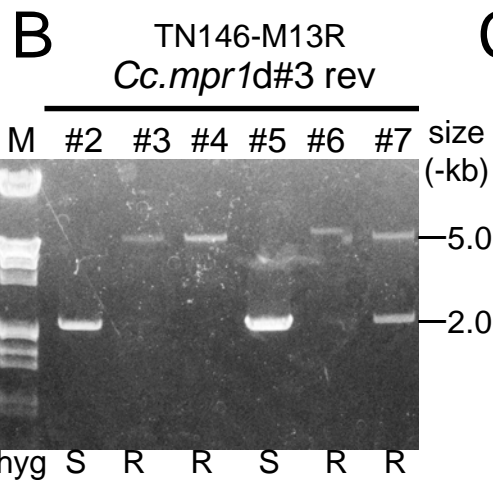
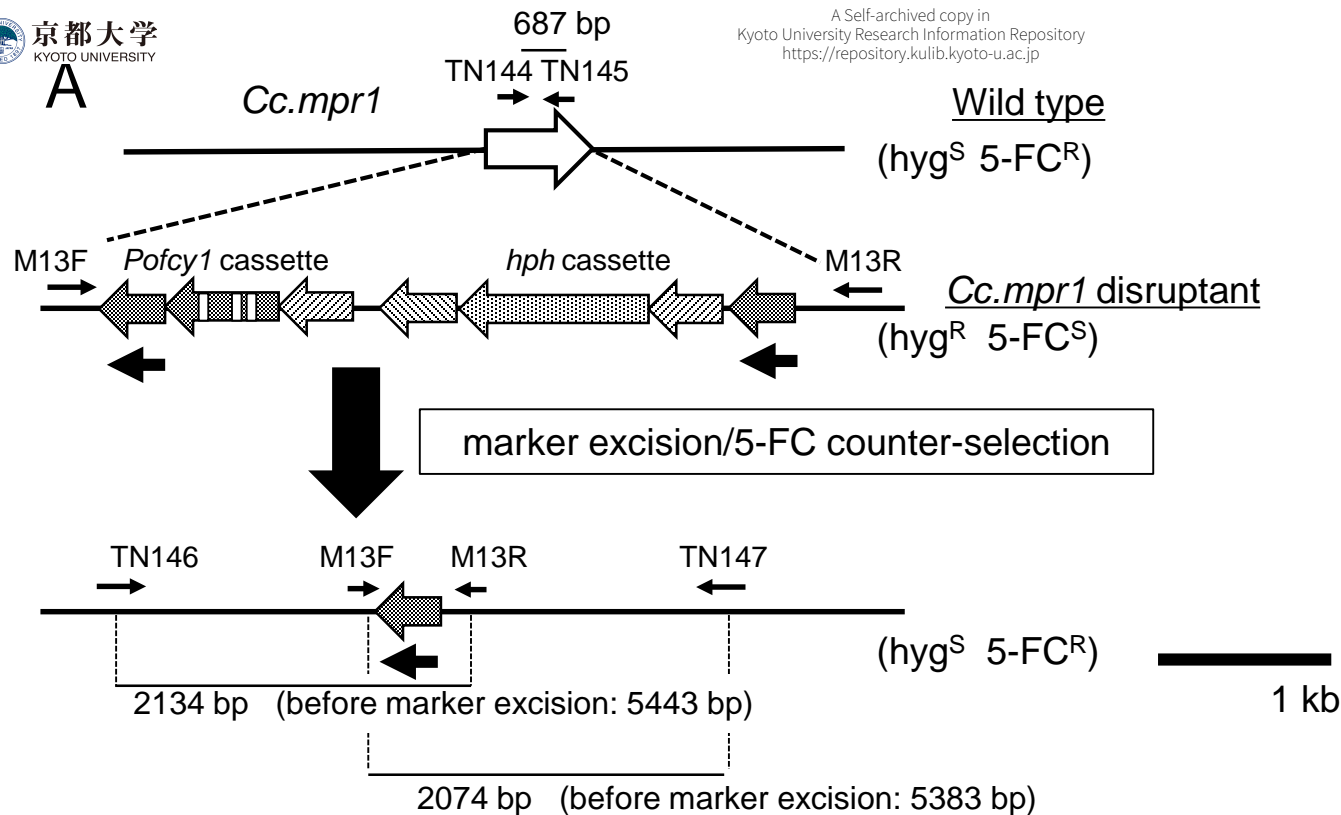


Fig. 4. Nakazawa et al.



**Table 1.** Strains used in this study.

Strain (species)	genotype/description	Source
326 ( <i>C. cinerea</i> )	<i>A43mut B43mut pab1-1</i>	P.J. Pukkila
PC9 ( <i>P. ostreatus</i> )	<i>A2 B1</i>	Larraya <i>et al.</i> (1999)
KF <sub>3</sub> #2 ( <i>C. cinerea</i> )	<i>A91 B91</i>	Muraguchi <i>et al.</i> (2003)
ku3-24 ( <i>C. cinerea</i> )	<i>A43mut B43mut pab1-1 ΔCc.ku70 (Flt<sup>R</sup>)</i> /a progeny of ku70dfltF <sub>2</sub> #92 x KF <sub>3</sub> #2	This study

**Table 2.** Primers used in this study.

TN40	ACCCTTTCCCCCAAATTTGGAAGC
TN41	ACCTTCTGGCATGACCTTTTGATGATCGC
TN84	ATGGAAAGCGCAGATCAACTGGG
TN85	CTAGTACATCTCTCCGATGTCTTCATACC
TN86	ATCTGCGCTTTCCATGCTGGGAACGCGAGGTCAGC
TN87	ATCTGCGCTTTCCATTTCCCTTTGCCCTCGGACGAG
TN88	GGAGAGATGTACTAGATGATTCGTTAGTTCTTTCC
TN101	ACGCCTCTGTCCCTTGTCTCCTC
TN102	ATCTCTCACCAGAACGAGGACTGC
TN103	TTCATTTAAACGGCTTCACGGGC
TN114	TATTGCGGGTGTAAGCTTAGAACGGCGG
TN115	AGGACTGGATCCAATGGTGGAACATATGAC
TN120	TCAAACCTTGATCTCTCTCCCGTC
TN121	ACGTCGTGACTGGGAGGCCAAACTCGGTTCTCTGTG
TN122	CCTGTGTGAAATTGTTGCCAAGACCAAAAGTGGGC
TN123	TAAGGACCGAAGAATGACCTGCTCG
TN144	TGTGCACCATCTCACGCTTCAG
TN145	TCGGCCTGCACCCGGAATTAAC
TN146	TTCTACTGTTATGCGGTTCTCTTTCCCC
TN147	ATTTTTTTGGGGCCCAGCAATGC
M13F	TCCCAGTCACGACGTTGTAAAACGACGG
M13R	ACAATTTACACAGGAAACAGCTATGACC